2002

Molecular Investigation of Genetic Variability Among Borrelia Infecting Ticks in Florida using Base Exision Sequence Scanning

Larry W. Martin
University of North Florida

Molecular Investigation of Genetic Variability Among *Borrelia* Infecting Ticks in Florida using Base Excision Sequence Scanning

Larry W. Martin

Faculty Sponsor: Dr. Kerry L. Clark
Assistant Professor of Health Science

Background

Changes in human ecology have contributed to the evolution of pathogenic microorganisms and the spread of disease throughout history. Diseases caused by infectious pathogens continue to challenge the public health and medical communities.[1] Despite previous claims of victory over infectious diseases by U.S. public health officials, infectious diseases remain the leading cause of morbidity and mortality worldwide.[2] In 1996, Nobel Prize-winning geneticist Joshua Lederberg wrote that concerning world health, "we have never been more vulnerable."[3] The number one public health priority is "concerted global and domestic surveillance and diagnosis of disease outbreaks and endemic occurrence. This must entail the installation of sophisticated laboratory capabilities at many centers now lacking them."[3]

Lyme disease (LD), the most commonly reported arthropod-associated illness in the U.S., is a multi-system tick-borne illness caused by the spirochete *Borrelia burfdorferi*.[4] In the eastern U.S. the agent is transmitted by the blacklegged tick, *Ixodes scapularis*,[5] and primarily maintained in nature by rodent reservoirs.[7] Although the majority of human cases has been concentrated in the northeastern U.S., hundreds have also been reported in southern states.[9] Some of these cases represent exposures that occurred elsewhere in the country. However, many were from endemic exposures, from which numerous indigenous cases have been confirmed in the Southeast.[10-15] Putative *B. burgdorferi* have been isolated from birds, rodents, and ticks in Florida, Georgia, South Carolina, and other southern states where *I. scapularis* is widely distributed and established.[16-20] In spite of these reports, however, some scientists are not convinced that endemic LD occurs in the southern U.S.

In the summer of 1999, Dr. K.L. Clark began testing rodent and tick DNA samples via polymerase chain reaction (PCR) assays for the presence of DNA of the LD bacteria. The results of these tests indicated that infection of rodents and ticks in Florida with the LD spirochete might be relatively high as compared to prevailing scientific belief. However, despite the sensitivity and specificity of DNA tests using the PCR, additional confirmation of the true identity of the agents infecting these rodents and ticks was warranted.

There is much genetic variation in the bacterial genospecies known to cause Lyme borreliosis, and several different closely related genospecies have been identified in the U .S. and other parts of the world.[21-24] Therefore, characterization of PCR-amplified DNA from the Florida rodent and tick samples was necessary to accurately identify the exact species/strain of *B. burgdorferi* infecting them, and to identify genetic variability among the samples.

Characterization of PCR-positive samples can be accomplished through a variety of molecular techniques, including DNA sequencing and phylogenetic analysis, but many of these methods are either very expensive, time-consuming, or technically challenging. Base Excision Sequence Scanning (BESS) is an easy, fast, and sensitive method that can be used to detect and identify exact base mutations present anywhere within a DNA fragment or to identify and type the species or strain of bacterium or virus DNA present in a biological sample.[25] For mutation detection, BESS involves less work than many other methods, while being capable of detecting nearly all of the mutations and identifying

exactly what the mutation is, rather than only indicating that a mutation exists somewhere within the sequence.

Purpose

The purpose of this project was to evaluate the use of the BESS technique for analyzing genetic variability and typing the species or strain of *B. burgdorferi* infecting rodents and ticks in Florida. It was expected that this technique would be identified as an easy, effective, and relatively inexpensive method for this purpose. Furthermore, it was anticipated that this procedure would become a standard to be used in ongoing and future vector-borne disease research.

Materials/Methods

PCR assays

DNA was extracted from individual blacklegged ticks and rodent ear tissue collected in Florida using the DNeasy™ Tissue Kit (Qiagen, Inc., Valencia, CA). A 5 uL sample of each DNA extract was used as template in amplification reactions using HotStarTaq™ Master Mix (Qiagen), which contained all the necessary components for the PCR except specific oligonucleotide primers. For increased sensitivity and specificity of amplification, a nested approach was used to amplify *B. burgdorferi*-specific fragments of the flagellin gene with primers derived by Johnson et al.[26] This gene was chosen as the target because of its known conservation among different strains/species of *B. burgdorferi* sensu lato. The initial (outer) reaction contained primers that amplify a 610 base pair portion of the flagellin gene of *B. burgdorferi* sensu stricto reference strains (B-31, JD-1). A 1 uL sample of the outer reaction product was used as template in a second (inner or nested) reaction that contained primers to amplify a 390 base pair sub-portion of the original fragment. Inner reaction products were electrophoresed on 2% agarose gels, and stained with fluorescent nucleic acid gel stain (either Sybr Green I or ethidium bromide). Stained products from tick or rodent samples were compared in size/molecular weight to those from *B. burgdorferi* reference strain positive control samples.

Various practices were routinely maintained to avoid and identify any carryover contamination of PCR's including the use of aerosol barrier pipet tips, exposing all pre-reaction tubes and tips to UV light, setting up all reactions in a dead air UV light PCR-dedicated chamber, liberal use of 10% bleach to disinfect surfaces, and the inclusion of negative water control samples for each group of amplified samples.

DNA sequencing and phylogenetic analysis

Prior to conducting BESS experiments, some PCR-positive tick and rodent samples were submitted to Dr. Bradley Schneider, Lyme Disease Vector Branch, Centers for Disease Control and Prevention, Fort Collins, CO, for confirmation DNA testing, DNA sequencing of amplified flagellin fragments, and phylogenetic analysis of flagellin sequence data. Several of these samples were included in the BESS experiments, since their exact flagellin gene fragment sequences were known.

BESS experiments

PCR-positive samples from Florida ticks, along with known *B. burgdorferi* sensu lato reference strains *(B. burgdorferi* sensu stricto, *B. andersonii,* and *B. bissettii),* were screened for genetic variability in attempts to type them to the exact *B. burgdorferi* species or strain using the BESS-T™ Base Reader Kit (Epicentre, Madison, WI). Resulting sequence profiles derived from infected Florida ticks were compared with those from the reference strain samples. DNA from samples that tested positive for the *B. burgdorferi* flagellin gene in previous PCR's, along

with appropriate positive and negative control samples, were used as template in nested amplification reactions using BESS dNTP mix, one 5'-biotin-end-labeled primer (flagellin gene inner reaction forward primer), and an unlabeled complementary primer (flagellin gene inner reaction reverse primer). PCR fragments thus amplified were then treated with BESS-T™ excision enzyme mix to digest all nucleotide bases except the deoxythymidine ("T") bases. This left products that, when sequenced, were essentially identical to "T -lane" sequence data derived from traditional sequencing methods. Cleavage products were separated on QuickPoint™ (Invitrogen, Carlsbad, CA) pre-cast mini-sequencing gels at 1,200V for 10 min. in a QuickPoint™ mini cell apparatus (Invitrogen). Reactions were then transferred to a positively charged nylon membrane (Tropilon-Plus™, Tropix, Bedford, MA), and prepared for chemiluminescent detection using the Phototope™-Star Detection Kit (NEB, Beverly, MA). Finally, sequenced products from the different samples were visualized by exposing the membrane to x-ray film and compared manually.

Results

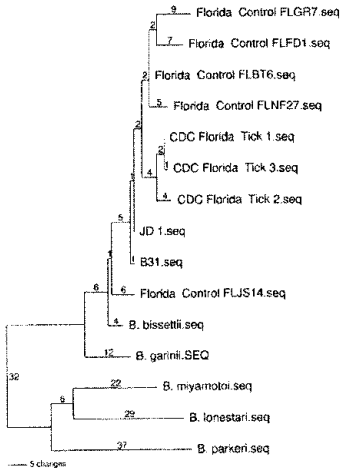
PCR assays

The results of nested PCR assays of small mammal and tick DNA samples for the presence of *B. burgdorferi* flagellin DNA are shown in Tables 1 and 2. Positive results were obtained for 4 species of small mammals from 2 sites in northeastern Florida, including golden mouse, rice rat, cotton mouse, and cotton rat (Table 1). Prevalence of infection among all animals tested from both UNF (38%) and Guana River State Park (40%) was almost identical. Positive results were also obtained from 2 species of ticks from 7 different sites in 5 counties in northern and central Florida (Table 2). The prevalence among the human biting *I. scapularis* from different sites ranged from 14% to 25%, and was 17% among all tested. The positive status of several of these samples from small mammals and ticks was confirmed via independent DNA testing by Dr. Schneider at the CDC.

DNA sequencing and phylogenetic analysis

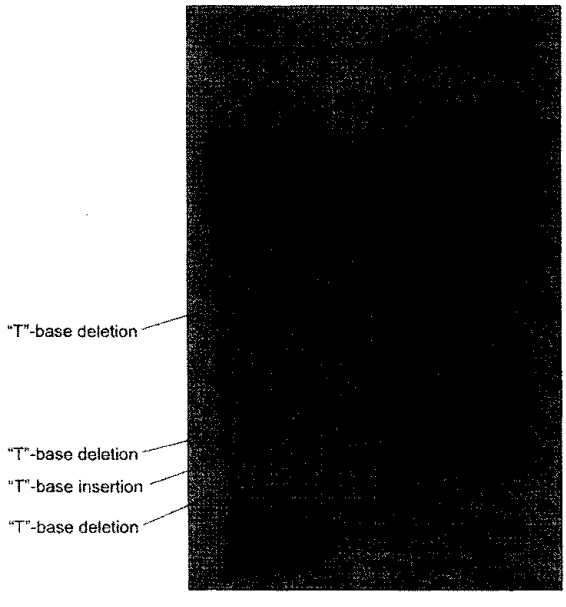
Flagellin gene fragments obtained from several Florida tick samples were sequenced, and some were included in a phylogenetic analysis to compare them with various *B. burgdorferi* sensu lato reference strains. Analysis of the fragment sequences identified numerous nucleotide differences among the Florida samples, and between the Florida samples and *B. burgdorferi* sensu lato reference strains. Figure 1 (courtesy of Bradley Schnieder, CDC, Ft. Collins, CO) shows a parsimony method phylogenetic tree depicting the results of that comparative analysis. It graphically displays the significant sequence divergence among the Florida samples and between the Florida samples and other *B. burgdorferi* sensu lato strains. One of the Florida samples (FLJS14) grouped with *B. bissettii* and likely represents a strain of that species. The other Florida samples formed two closely related groups that were most like (although fairly different in sequence from) *B. burgdorferi* sensu stricto strains (B-31, JD-l).

Figure 1. Parsimony phylogenetic tree for PCR-amplified flagellin gene sequences



Tick 1 =FLFG2m
Tick 2 = FLNF1f
Tick 3 = FLFG3m

Figure 2. BESS-T sequence data for *B. burgdorferi* sensu lato reference samples and Florida tick samples: Lanes 1 and 2, *B. burgdorferi* sensu stricto (JD-1 strain); Lanes 3 and 4, *B. burgdorferi* sensu stricto (SCCH-4, cotton mouse in SC); Lanes 5 and 6, *B. andersonii* (MOK-1C, tick in MO); Lanes 7 and 8, *B. bissettii* (SCW-30H, woodrat in SC); Lanes 9 and 10, *B. bissettii* (FLJS14, FL tick; Lanes 13 and 14, FLNF 27, FL tick; Lanes 15 and 16, FLFD1, FL tick.



BESS experiments

Numerous BESS experiments were conducted in efforts to refine the procedure. Various *B. burgdorferi* sensu lato reference strain samples were included for positive controls to compare with the results from "unknown" strains derived from Florida ticks. Figure 2 shows typical BESS- T sequence data obtained with the methods described above. The intensity of the T-nucleotide bands varied due to variation in the level of amplification in the PCR step. This was likely due to variation in the number of copies of target DNA present among the initial DNA extracts. The sequence data was difficult to compare manually due to poor resolution. The only manually detectable sequence differences among the samples were located in lanes 5 and 6, which contained replicates of *B. andersonii* (MOK -IC strain derived from an *Ixodes dentatus* tick in Missouri). A single T-base insertion, and 3 different T-base deletions could be detected within the sequence of *B. andersonii* (as compared to the other samples), while no differences among all the other samples were manually observable.

Table 1. Prevalence of *Borrelia burgdorferi* sensu lato flagellin gene DNA among small mammals collected in Florida, 1999.

County	Site	Virginia Opossum	Flying Squirrel	Golden Mouse	Rice Rat	Cotton Mouse	Cotton Rat	Total
Duval	UNF	0/1*	0/1	1/2 (50)	-	10/28 (36)	2/2 (100)	13/34 (38)
St. Johns	Guana River State Park	-	-	0/1	3/3 (100)	1/6 (17)	-	4/10 (40)
Total		0/1	0/1	1/3 (33)	3/3 (100)	11/34 (32)	2/2 (100)	17/44 (39)

* Number of PCR positive animals/number tested = % each species

Table 2. Minimum PCR-positive prevalence of *Borrelia burgdorferi* sensu lato flagellin gene DNA among pools of ticks collected in Florida, 1999.

County	Site	Tick species	Number tested	Minimum number positive	Minimum prevalence (%)*
Duval	Ft. George Island	*I. scapularis*	36	6	17
Duval	UNF	*I.affinis*	4	1	25
		I. scapularis	14	2	14
Hamilton	Stephan Foster State Folk Culture Center	*I. scapularis*	35	6	17
Lake	Alexander Springs	*I. affinis*	1	1	100
		I. scapularis	4	1	25
Lake	River Forest	*I. affinis*	1	1	100
		I. scapularis	12	2	17
Columbia	Ichetuknee Springs State Park	*I. scapularis*	6	1	17
Volusia	Tomoka State Park	*I. scapularis*	18	3	17

* Minimum prevalence for all *I. affinis*: 3/6 = 50%; minimum prevalence for *I. scapularis*: 21/125 = 17%; ticks were tested in pools containing DNA from several individuals; it was estimated that each positive pool contained only 1 positive tick.

Discussion

The data obtained from this study verify the presence of *B. burgdoferi* sensu lato among ticks and small mammals at several sites in northeastern and north central Florida. The distribution of the sites from which positive ticks were obtained indicates that the organism is widely dispersed. The estimated prevalence of infection among small mammals (39%) and adult blacklegged ticks (17%) also suggest a fairly intense enzootic transmission cycle at the localities studied. The results of DNA sequencing and phylogenetic analysis show that at least two *B. burgdorferi* sensu lato genospecies, *B. bissettii* and another strain most similar to *B. burgdorferi* sensu stricto, are enzootically present in Florida. The specific identity of most of the *B. burgdorferi* flagellin-positive Florida samples remains to be determined. Although, in their flagellin gene sequences they were most similar to sensu stricto strains, they were significantly divergent to suggest that they may represent at least 1 previously undescribed *B. burgdorferi* sensu lato genospecies. Additional evidence to support this divergence was our inability amplify portions of other target genes from most Florida samples with primers commonly used to identify *B. burgdorferi*. These target genes included the outer surface protein A, 66-kDa antigen, and 5S-23S intergenic spacer region genes of *B. burgdorferi*.

The lack of resolution of BESS-T - derived sequence data using the methods described herein did not allow for identification of all known sequence differences among the samples compared. In order to most effectively utilize the BESS-T method to identify single nucleotide base mutations, deletions, and insertions among samples analyzed, it will be necessary to combine the technique with use of an automated DNA sequencing system. With the high resolution capabilities of automated sequencing and detection systems, which use fluorescent dyes and laser-optic detection methods, combined with available computerized sequence alignment and comparison programs, the BESS-T methodology may prove to be a relatively simple, inexpensive, and highly effective way to

identify, compare, and type *Borrelia* strains. In the future, additional *B. burgdorferi* flagellin-positive samples from Florida will be sequenced and compared with the sequences of various known strains of *Borrelia*.

Furthermore, experiments will be conducted to identify other gene targets besides the flagellin gene for consistent amplification, and phylogenetic comparison of Florida *Borrelia* species.

Work Cited

1. Berkelman, R.L. and J.M. Hughes. 1993. The conquest of infectious diseases: who are we kidding? *Ann. Intern. Med.* 119: 426-428.

2. Global Health Situation and Projections. 1992. Geneva: World Health Organization.

3. Lederberg, I. 1996. Infection emergent. *JAMA*

4. Johnson, R.C., G P. Scmid, F.W. Hyde, A.G. Steigerwalt, and D.J. Brenner. 1984. *Borrelia burgdorferi* sp. nov.: etiologic agent of Lyme disease. *Int. J.Syst. Bacteriol.* 34: 496-497.

5. Burgdorfer, W., A.G. Barbour, S.F. Haybes, J.L. Benach, E. Grunwaldt, and J.P. Davis. 1982. Lyme disease-a tick-borne spirochetosis? *Science* 256: 1439-1442.

6. Burgdorfer, W., A.G. Barbour, S.F. Hayes, O. Peter, and A. Aeschlimann. 1983. Erythema chronicum migrans- a tick-borne spirochetosis. *Act. Trop* 40: 79-83.

7. Levine, J.F., M.L. Wilson, and A. Spielman. 1985. Mice as reservoirs of the Lyme disease spirochete. *Am. J. Trop. Med. Hyg* 34: 355-360.

8. Mather, T.N., M.L. Wilson, S.I. Moore, J. M.C. Ribeiro, and A. Spielman. 1989. Comparing the relative potential of rodents as reservoirs of the Lyme disease spirochete *(B. burgdorferi). Am. J. Epidemiol* 130: 143-150.

9. Centers for Disease Control and Prevention. 2001. Lyme disease-United States, 1999. *MMWR* 50: 181-185.

10. Mullen, G.R. and J. Piesman. 1987. Serologically substantiated cases of Lyme disease and potential tick vectors in Alabama. *AL J. Med. Sci.* 24: 306- 307.

11. Centers for Disease Control and Prevention. 1989. Lyme disease-United States, 1987 and 1988. MMWR. 38: 668-672.

12. Ciesielski, C.A., L.E. Markowitz, R. Horsley, A.W. Hightower, H. Russell, and C.V. Broome. 1989. Lyme disease surveillance in the United States, 1983-1986. *Rev. Infect. Dis.II*: S1435- S1441.

13. Centers for Disease Control and Prevention. 1991. Lyme disease surveillance-United States, 1989-1990. *MMWR* 40: 417-421.

14. Levine, J.F., C.S. Apperson, R.A. Spriegel, W.L. Nicholson, and C.J.. Staes. 1991. Indigenous cases of Lyme disease diagnosed in North Carolina. *South. Med. J.* 84: 27-31.

15. Oliver, J.H., Jr. 1996. Lyme borreliosis in the southern United States: a review. *J. Parasitol* 82: 926-935.

16. Durden, L.A., R.G. McLean, J.H. Oliver, Jr., S.R. Ubico, and A.M. James. 1997. Ticks, Lyme disease spirochetes, trypanosomes and antibody to encephalitis viruses in wild birds from coastal Georgia and South Carolina. *J. Parasitol* 83: 1178-1182.

17. Oliver, J.H., Jr., K.L. Clark, F.W. Chandler, Jr., L. Tao, A.M. James, C.W. Banks, L.O. Huey, A.R. Banks, D.C. Williams and L.A. Durden. 2000. Isolation, cultivation, and characterization of *Borrelia burgdorferi* from rodents and ticks in the Charleston area of South Carolina. J. Clin. Microbiol. 38: 120-124.

18. Oliver, J.H., Jr., F.W. Chandler, Jr., M.P. Luttrell, A.M. James, D.E. Stallknecht, *burgdorferi* in the United States. *J. Infect. Dis.* 175: 98-107.

19. Oliver, J.H., Jr., F.W. Chandler, Jr., A.M. James, F.H. Sanders, Jr., .J..Hutcheson, L.O..Huey, B.S. McGuire and R.S. Lane. 1995. Natural occurrence and characterization of the Lyme disease spirochete, *Borrelia burgdorferi,* in cotton rats *(Sigmodon hispidus)* from Georgia and Florida. *J. Parasitol* 81: 30-36.

20. Piesman, J., K.L. Clark, M.C. Dolan, C.M. Happ and T.R. Burkot. 1999. Geographic survey of the principal vectors of Lyme disease in North America *(Ixodes scapularis* and *Ixodes pacificus)* for infection with the etiologic agent, *Borrelia burgdorferi. J.Vector Ecol* 24: 91-98.

21. Barbour, A.G., R.H. Heiland, and T.R. Howe. 1985. Heterogeneity of major proteins in Lyme disease borreliae: a molecular analysis of North American and European isolates. *J. Infect. Dis.* 152: 507-516.

22. Mathiesen, D.A., J.H. Oliver, Jr., C.P. Kolbert, E.D. Tullson, B.J.B. Johnson, G.L. Campbell, P.D. Mitchell, K.D. Reed, S.R. Telford III, J.F. Anderson, R.S. Lane, and H. Persing. 1997. Genetic heterogeneity of *Borrelia burgdorferi* in the United States. *J. Infect. Dis.* 175: 98-107.

23. Park, K., W. Chang, and T.G. Schwan. The Identification and characterization of Lyme disease spirochetes, *Borrelia burgdorferi* sensu lato isolated in Korea. *J. Clin. Microbiol* 31: 1831- 1837.

24. Postic, D., M.V. Assous, P.A.D. Grimont, and G. Baranton. 1994. Diversity of Borrelia burgdorferi sensu lato evidenced by restriction fragment length polymorphism of rrf (5S)-rrl (23S) intergenic spacer amplicons. *Int. J. Syst Bacteriol* 44: 743-752.

25. Charrel, R.N., and L.J. Chandler. 1999. Epidemiological typing of viruses using the BESS-T Base Reader Kit: identification of genetic variation among St. Louis encephalitis virus isolates using an ABI automated DNA sequencer. *Epic. For.* 6 (2): 9-13.

26. Johnson, B.J. B., C.M. Happ, L. W. Meyer, and J.Piesman. 1992. Detection of *Borrelia burgdorferi* in ticks by species-specific amplification of the flagellin gene. *Am J. Trop. Med. Hyg* 47: 730-741.